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The potential role of tissue zonulin levels as diagnostic biomarkers in colon adenocarcinomas

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Abstract

Distinguishing between benign and malignant masses during endoscopic examination of colon masses is critical; however, predictive parameters other than lesion appearance are lacking. This study aimed to compare the zonulin levels in colorectal cancer tissues with those in normal colon tissues to ascertain their diagnostic value. Our study included 108 patients in the study group whose colonoscopy revealed a mass in the colon and whose biopsy revealed adenocarcinoma, and 60 healthy individuals in the control group with normal colonoscopy findings. Three samples were collected: 1 cancerous tissue, 1 healthy tissue (approximately 10–15 cm from the cancerous tissue in the study group), and 1 healthy colon tissue in the control group. These samples were stored at –80°C and their zonulin levels were compared to determine their correlation with clinical variables. The sample size was calculated with a 95% confidence interval and 80% power. As a result of this calculation, 108 patients and 60 control group participants were identified. Control group selection criteria were determined as follows: Healthy individuals matched to the study group in terms of age and gender. The median zonulin levels in the study group were 60.98 ng/mL in cancerous tissues, 27.98 ng/mL in noncancerous tissues, and 15.80 ng/mL in the control group. Zonulin levels in cancerous tissues were significantly higher than those in healthy tissues (60.98 vs 27.98, respectively, $P < .001$) and control tissues (60.98 vs 15.80, respectively, $P < .001$). Significantly higher zonulin levels were noted in both healthy and tumor tissues of female patients than in those of male patients (30.30 vs 22.45, $P < .001$ and 63.17 vs 49.36, $P = .009$, respectively). The median zonulin level in colorectal adenocarcinoma tissues was significantly higher than that in the healthy colon tissues of the control group and normal colon tissues of patients with cancer. If corroborated by future studies, zonulin levels may serve as a potential predictive marker with diagnostic value.

Abbreviations: CRC = colorectal cancer, CT = chemotherapy, ELISA = enzyme-linked immunosorbent assay, PBS = phosphate-buffered saline.

Keywords: adenocarcinoma, biomarker, colonoscopy, colorectal cancer, diagnosis, tissue zonulin level, tumor

1. Introduction

Colorectal cancer (CRC) has a high mortality rate when detected at advanced stages. However, early diagnosis significantly prevents its progression. Over the past 2 decades, the age-standardized mortality rate has decreased due to early diagnosis, widespread use of appropriate treatment methods, and a declining incidence.[1] Among cancer types, CRC ranks 3rd in terms of incidence and 2nd in cancer-related deaths.[2] Various effective techniques for early diagnosis include stool-based (fecal occult blood, immunochemical, and DNA tests), endoscopic (optical colonoscopy and flexible sigmoidoscopy), and imaging techniques (computed tomography/magnetic resonance colonography, capsule endoscopy, and barium enema studies).

Zonulin has been identified as a protein that modulates intestinal permeability by dissolving intercellular tight junctions, the most apical junctional complex of the paracellular pathway. It has been shown to be a potential biomarker for autoimmune, nervous system, and neoplastic diseases. Zonulin may also serve as an early diagnostic tool and therapeutic target for the treatment of these conditions, including CRC.[3,4] Recently, the relationship between the intestinal barrier, microbiota, and the immune system has gained increasing attention. The intestinal tract is the largest immune organ in the human body and is colonized by microbiota consisting of approximately 100 trillion microorganisms.[5] The intestinal epithelial barrier, composed of a continuous single layer of epithelial cells, functions by closing the paracellular pathway with tight junction proteins. This barrier is selectively permeable, allowing the absorption of water, electrolytes, and nutrients while blocking the passage of antigens, gut microbiota, and toxins.[6] Some bacterial toxins have been shown to coordinate the carcinogenic inflammatory cascade by targeting

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The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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colonic epithelial cells.[7] An increasing number of studies have found changes in zonulin levels in the case of "leaky gut"; however, whether these changes are present in different pathological conditions, including CRC, remains unclear.[8] Some studies[9,10] have shown an association between serum zonulin levels and CRC; however, to our knowledge, the relationship between tissue zonulin concentrations and CRC remains elusive.

Our primary hypothesis is that zonulin levels are increased in CRC tissues. We further hypothesize that these increased levels correlate with the clinical features of CRC, tumor localization, differentiation and metastatic status. Although the relationship between serum zonulin levels and CRC has been previously investigated, the relationship between tissue zonulin concentrations and CRC remains unclear. This gap in the literature warrants further research to determine the diagnostic potential of tissue zonulin levels in CRC.

Other tissue parameters will assist in pathological evaluation and provide rapid results for the description of lesions with a colonoscopic view suggestive of malignancy. This study's objective is to find the method based on the level of tissue zonulin for CRC diagnosis. In this study, the research objectives include comparing the amount of zonulin protein expression in colon cancer tissues and in normal colon tissues and investigating the relation between tissue zonulin and clinical parameters of CRC patients, as well as assessing the possible use of tissue zonulin as a biomarker for CRC diagnosis. In relation to such objectives, this study tries to contribute to the early detection of CRC and enhanced diagnostic reliability, which can help enhance treatment and survival rates in patients.

2. Materials and methods

2.1. Study design and sampling

In this cross sectional study, we compared tissue zonulin levels in the normal and tumor regions of the bowel of patients diagnosed with CRC with those in the normal bowel regions of a control group. The sample size was calculated with a 95% confidence interval and 80% power. As a result of this calculation, 108 patients and 60 control group participants were identified. These numbers represent an adequate sample size to obtain statistically significant results. Patients with CRC were divided into 2 groups based on tumor location: Right CRC (if the tumor was located proximal to the splenic flexure) and left CRC (if it was located distal to the splenic flexure). Zonulin levels in cancerous and normal tissues, 10 to 15 cm from the tumor were studied and compared with colon tissue zonulin levels in healthy subjects. All colonoscopies were performed by the same endoscopist with 12 years of experience.

2.2. Patient selection and ethical approval

A total of 108 patients who presented to our university hospital between July 2020 and June 2022 and were diagnosed with CRC using tissue biopsy during colonoscopy were included in the study. The exclusion criteria were set as follows and the rationale was explained: Uncontrolled diabetes mellitus (due to the potential impact of glycemic control on zonulin levels), advanced heart disease (New York Heart Association Class III–IV, due to possible effects of circulatory impairment on the intestinal barrier), chronic renal failure (estimated glomerular filtration rate <30 mL/min/1. 73 m^2, because uremia may affect intestinal permeability), chronic liver disease (Child-Pugh B or C, because liver dysfunction may affect zonulin metabolism), inflammatory and autoimmune diseases (because of the direct effects of these diseases on the intestinal barrier), and morbid obesity (body mass index ≥40 kg/m^2, because of the known effects of obesity on intestinal permeability). These diseases were considered as factors that could affect zonulin levels and were excluded to increase the reliability of the results. Patients with cancer types other than adenocarcinoma detected by colon biopsy were excluded from the study.

Control group selection criteria were determined as follows: Healthy individuals matched to the study group in terms of age and gender, individuals with normal colonoscopy findings, individuals without any known chronic disease, individuals without a history of antibiotic use in the last 6 months, and individuals without regular medication use. Sixty patients with normal colons who underwent colonoscopy for various indications were included in the control group. No chronic smokers or individuals with alcohol use disorder were included in either the case or control groups. No abnormalities were observed in the patients' blood biochemistry tests that may have affected the study results.

The patients in both groups voluntarily participated in the study, and informed consent was obtained. The study was approved by the ethical board of our institution and was conducted according to the ethical guidelines of the Declaration of Helsinki, as revised in 2013 (Ethics committee date/no: 02.07.2020/544).

2.3. Collection and preservation of samples

During the colonoscopy (SonoScape HD500; China) of the study group, 3 to 4 tissue samples were collected from areas suspected of harboring cancerous tissue, and an additional 3 to 4 tissue samples were taken from normal-looking sites, 10 to 15 cm away from the initial areas. The following procedures were followed during the collection of tissue samples: Biopsy forceps were prepared sterile, specimens were taken from the mucosal layer and care was taken not to damage the submucosal tissue, specimens were placed in sterile tubes immediately upon collection, and tubes were placed in precooled transport containers to avoid temperature changes that could affect zonulin levels.

The samples were immediately placed in a freezer at –80°C (Nüve DF 490; Turkey) and preserved without processing. The reason for storing samples at –80°C is to minimize degradation of the zonulin protein. This temperature minimizes enzymatic activity and protein degradation. The following measures were taken to prevent degradation during processing: The cold chain was maintained until the samples were transferred to the laboratory, thaw-freeze cycles were avoided, and samples were stored for a maximum of 3 months until analysis.

Similarly, 3 to 4 samples were obtained from the colon segment of the control group patients that appeared normal during colonoscopy, and the samples were stored in the same freezer at –80°C without further processing.

2.4. Tissue homogenisation and protein extraction

Next, 5 mg of tissue was weighed and suspended in ice-cold phosphate-buffered saline (PBS) at a 1:9 ratio. For the preparation of PBS, we used Sigma-Aldrich P4417 PBS tablets (Sigma-Aldrich, St. Louis). One tablet was dissolved in 200 mL of deionized water to yield a 0.01 M phosphate buffer solution (pH 7.4) containing 0.0027 M potassium chloride and 0.137 M sodium chloride. The tissue was then disrupted and homogenized using a Qiagen TissueLyser. Homogenization was performed at 30 Hz for 2 minutes with 5 mm stainless steel beads. The sample was sonicated, using a Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) with 30% amplitude for 3 10-second pulses with 10-second cooling intervals on ice, and the cells were completely lysed. No additional lysis buffer was used as the mechanical disruption in PBS was sufficient for our

Table 1

Demographic data, tissue zonulin levels comparison, and ROC analysis results for control and study groups.

Variable	Control group (n = 60)	Study group (n = 108)	*P*
Age (yr)			
Mean ± SD	61.3 ± 7.3	62.4 ± 12.6	.354
Median (IQR)	63.0 (56.3–67.0)	65.5 (49.3–71.8)	
Sex, n (%)			
Female	36 (60.0)	78 (72.2)	.104
Male	24 (40.0)	30 (27.8)	
Zonulin levels (ng/mL)	Healthy tissues	Healthy tissues	Tumor tissues
Mean ± SD	16.5 ± 4.3	29.8 ± 12.1	67.9 ± 27.9
Median (IQR)	15.8 (14.2–18.1)	28.0 (23.1–33.1)	61.0 (47.3–88.2)
ROC analysis results			
AUC (95% CI)	0.72 (0.64–0.80)	0.93 (0.89–0.97)	
Cutoff value (ng/mL)	19.5	42.5	
Sensitivity (%)	68.3	89.8	
Specificity (%)	71.7	88.3	
PPV (%)	70.7	92.4	
NPV (%)	69.4	84.1	

AUC = area under the curve, CI = confidence interval, IQR = interquartile range, NPV = negative predictive value, pa = *P*-value for age comparison, pb = *P*-value for sex comparison, PPV = positive predictive value, ROC = receiver operating characteristic, SD = standard deviation.

analysis. The homogenates were centrifuged for 5 minutes at 5000 g at 4°C, and the supernatants were collected.

2.5. Zonulin measurement

Technical details about the enzyme-linked immunosorbent assay (ELISA) kit (Human Zonulin ELISA Kit; Catalog no: MBS3802140; MyBioSource, Inc., San Diego) used for the measurement of zonulin: Sensitivity 0.1 ng/mL, measuring range 0.625 to 20 ng/mL, intra-assay CV <8%, inter-assay CV <10%, and high specificity for human zonulin, no cross-reactivity with other proteins. To ensure the accuracy of the measurements, the following calibration procedures were performed: A standard curve was generated in each measurement series, control samples were used at high, medium and low concentrations, all samples were run in duplicate and mean values were used, and the results were validated with quality control materials provided with the kit. Tissue zonulin concentrations in the supernatants were measured using ELISA with commercial kits.

2.6. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 20.0 software (IBM Corp., Armonk). For numerical data, descriptive statistics were presented as the mean ± standard deviation and median with interquartile range, and categorical data as frequency (n) with percentage (%). The assumptions and rationale for the use of statistical tests are as follows: Pearson's chi-square test was used to examine the relationship between categorical variables and the assumption of expected frequencies >5 was checked. Shapiro–Wilk and Kolmogorov–Smirnov tests were used to test the conformity of the data to normal distribution, and if $P > .05$, the data were considered normally distributed. Wilcoxon signed-rank test was therefore utilized in comparing the dependent groups with the assumption that the data collected were continuous and that the patients were paired. To compare the 2 independent groups, the Mann–Whitney *U* test was conducted and to maintain the condition of continuity and non-normality of the data, it was achieved. Serum cortisol was used to compare more than 2 independent groups by clients received different modality of exercise, while the assumption of continuous and non-normal distribution of data was checked by Kruskal–Wallis test. Pair wise comparisons after Kruskal–Wallis test were done using Dunn's test and to correct for multiple errors, Bonferroni correction was used. The inter-variable association of 2 items was tested using Spearman's correlation where it was assumed that the data at least was ordered. $P < .05$ was considered to indicate statistical significance.

3. Results

In this study, we compared zonulin levels in tissues from CRC patients and healthy controls. The results showed that zonulin levels in tumor tissues of CRC patients (median 60.98 ng/mL) were significantly higher than both healthy tissues of patients (median 27.98 ng/mL) and healthy tissues of the control group (median 15.80 ng/mL; $P < .001$). Furthermore, zonulin levels in healthy tissues of CRC patients were also significantly higher than in the control group ($P < .001$). These findings suggest that zonulin levels may be a potential biomarker in the diagnosis of CRC. The results of receiver operating characteristic analysis also support this view, with a sensitivity of 89.8% and specificity of 88.3% when the threshold value for tumor tissue was 42.5 ng/mL. Regarding demographic data, no significant difference was observed between the study and control groups in terms of age and gender ($P > .05$; Table 1 and Fig. 1).

Gender and age distribution of zonulin levels in subjects also revealed the following differences. In the control group there were no differences between genders (16.05 ± 3.33 ng/mL in women and 15.66 ± 2.86 ng/mL in men, $P = .330$). However, in the study group, we observed differences in zonulin levels between females and males in healthy tissues (30.30 ± 3.23 ng/mL in women and 22.45 ± 3.07 ng/mL in men, $P < .001$) and in tumor tissues (72.00 ± 29.39 ng/mL in women and 57.06 ± 20.30 ng/mL in men, $P = .009$). It should be noted that our study cohort had an unbalanced gender distribution (78 females vs 30 males), which constitutes a limitation of our study. This imbalance might have affected the reliability of gender-related conclusions, and our findings regarding gender differences should be interpreted cautiously and verified in future studies with more balanced gender representation. In terms of age relationships, the control group showed minimal correlation with age ($r = 0.15$). This study revealed that zonulin levels in healthy tissues were slightly higher in older cancer patients compared to younger individuals ($r = 0.28$) but zonulin levels were lower in tumor tissues as age increases ($r = -0.22$). Therefore, there is a need for more detailed studies of the relationship between zonulin levels and clinical parameters of CRC diagnosis and prognosis, with consideration for gender and age (Table 2 and Fig. 2).

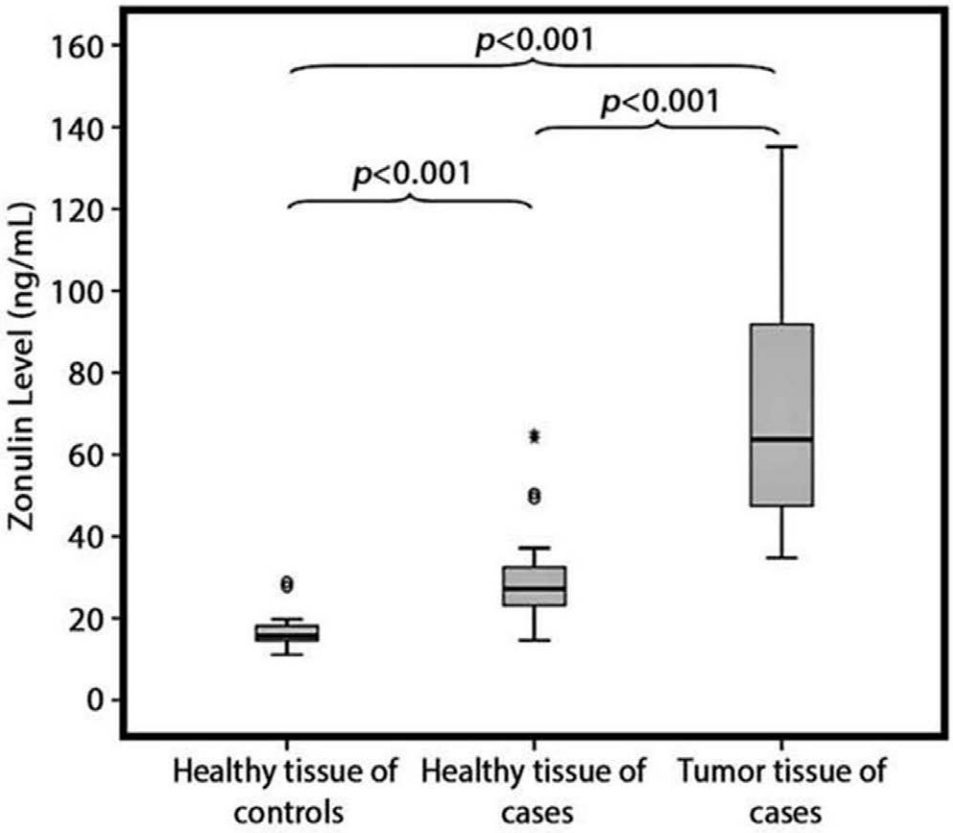

Figure 1. Box plots of tissue zonulin levels in different sample types. The chart displays median values and interquartile ranges of zonulin concentrations (ng/mL) in healthy control tissues, noncancerous tissues from CRC patients, and tumor tissues from CRC patients. Statistical significance ($P < .001$) is shown between all 3 groups. CRC = colorectal cancer.

Table 2

Comparison of tissue zonulin levels between females and males and correlation with age.

	Sampling site	Zonulin level (ng/mL)		
Group	**Females**	**Males**	**pa**	**Correlation with age (*r*)b**
Control group	Healthy tissues			0.330
Mean ± SD	17.25 ± 4.79	15.49 ± 3.22		
Median (IQR)	16.05 (14.41–18.91)	15.66 (12.87–18.02)		
Study group	Healthy tissues			<0.001
Mean ± SD	32.86 ± 12.59	21.96 ± 5.30		
Median (IQR)	30.30 (25.15–36.06)	22.45 (17.19–26.30)		
Tumor tissues			.009	–0.22
Mean ± SD	72.00 ± 29.39	57.06 ± 20.30		
Median (IQR)	63.17 (49.69–91.88)	49.36 (40.28–72.70)		

IQR = interquartile range, pa = *P*-value for comparison between females and males, *r* = correlation coefficient with age, SD = standard deviation.

In our study, tissue zonulin levels were compared according to clinical characteristics and TNM staging of CRC patients (Table 3). Higher zonulin levels were observed in right colon tumors (32.20 ± 5.19 ng/mL) compared to left colon tumors (29.15 ± 13.36 ng/mL), while higher zonulin levels were detected in well-differentiated tumors (36.27 ± 13.85 ng/mL) compared to intermediate (24.49 ± 7.48 ng/mL) and poorly differentiated (26.30 ± 1.56 ng/mL) tumors. According to metastasis status, zonulin levels were 27.85 ± 6.89 ng/mL in nonmetastatic patients, 29.31 ± 14.61 ng/mL in patients with local lymph node metastasis and 37.78 ± 19.11 ng/mL in patients with distant metastasis (Fig. 3). In TNM staging, zonulin levels were 26.45 ± 5.32 ng/mL in Stage I patients. These findings suggest that tissue zonulin levels may be associated with clinical features of CRC, presence of metastasis and disease stage, and zonulin levels tend to increase especially in the presence of metastasis and advanced stages.

In our study, zonulin levels were compared in detail between right and left colon cancers. In healthy tissues, significantly higher zonulin levels were observed in patients with right colon cancer (32.20 ± 5.19 ng/mL) than in patients with left colon cancer (29.15 ± 13.36 ng/mL; $P = .003$). No statistically significant difference was found in tumor tissues (right colon: 56.92 ± 9.96 ng/mL, left colon: 70.98 ± 30.52 ng/mL, $P = .196$). However, when the ratio of tumor tissue zonulin levels to healthy tissue levels was analyzed, a higher increase was detected in left colon cancers (mean ratio: 2.43) compared to right colon

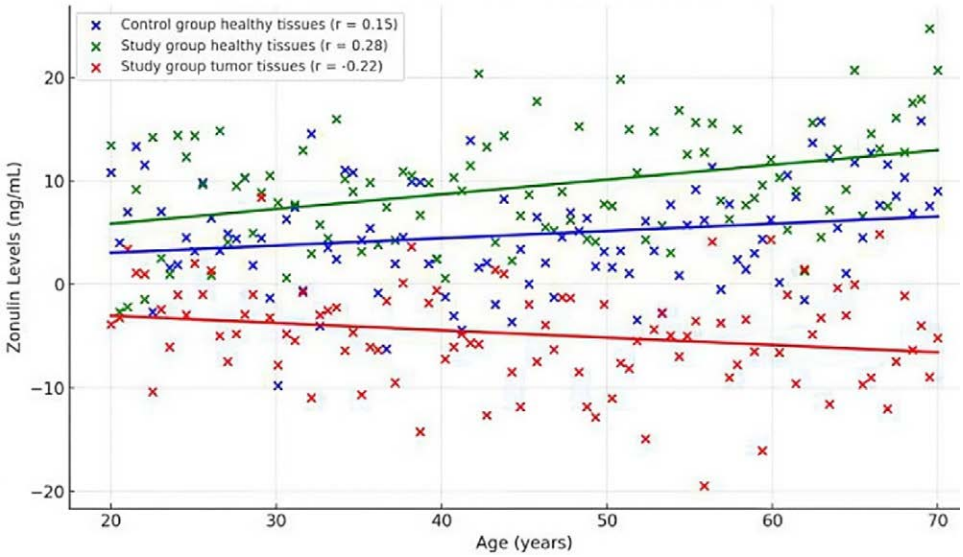

Figure 2. Relationship between age and zonulin levels in different tissue types. Scatter plot showing correlation between age (yr) and zonulin levels (ng/mL) with regression lines. Blue points represent control group healthy tissues ($r = 0.15$), green points represent study group healthy tissues ($r = 0.28$), and red points represent study group tumor tissues ($r = -0.22$).

Table 3

Comparison of tissue zonulin levels in patients with colorectal cancer by clinical characteristics and TNM staging.

	n (%)	
	Healthy tissues	Study group zonulin levels (ng/mL)
Characteristics	Mean ± SD	Median (IQR)
Tumor side		
Right-sided	24 (22.2)	32.20 ± 5.19
Left-sided	84 (77.8)	29.15 ± 13.36
Differentiation		
Well	48 (44.4)	36.27 ± 13.85
Moderate	54 (50.0)	24.49 ± 7.48
Poor	6 (5.6)	26.30 ± 1.56
Metastasis		
Nonmetastatic	66 (61.1)	27.85 ± 6.89
Local lymph node metastasis	24 (22.2)	29.31 ± 14.61
Distant metastasis	18 (16.7)	37.78 ± 19.11
TNM stage*		
Stage I	18 (16.7)	26.45 ± 5.32
Stage II	48 (44.4)	28.32 ± 7.15
Stage III	24 (22.2)	29.31 ± 14.61
Stage IV	18 (16.7)	37.78 ± 19.11

IQR = interquartile range, SD = standard deviation, TNM = tumor, node, metastasis.
*TNM staging according to the American Joint Committee on Cancer (AJCC) 8th Edition.

cancers (mean ratio: 1.77; $P = .015$). These findings suggest that the localization of CRC may affect zonulin levels and tumor to healthy tissue ratios (Table 4).

In our study, zonulin levels were analyzed according to the treatment modalities applied in CRC patients. As shown in Figure 4, zonulin levels in both healthy tissues and tumor tissues differed according to the treatment modality. Lower zonulin levels were observed in patients who underwent a combination of surgery and chemoradiotherapy compared to patients who underwent surgery alone. In patients receiving palliative treatment, higher zonulin levels were detected, especially in tumor tissue. The highest zonulin levels were observed in patients receiving chemotherapy (CT) alone. These findings suggest that treatment modality may affect zonulin expression and zonulin levels may be a potential biomarker for assessing treatment response (Fig. 4).

4. Discussion

Zonulin, identified as the sole physiological modulator of intercellular tight junctions discovered to date, is a protein involved in the regulation of both epithelial and endothelial barrier functions, and its role in health and disease remains an object of active research.[11] Previously, the identification of zonulin levels has proven pivotal in improving the diagnostic accuracy of hepatocellular carcinoma, even distinguishing it from liver cirrhosis when combined with alpha-fetoprotein. Zonulin serves as a potential indirect indicator of proinflammatory factors, such as tumor necrosis factor-alpha, Interleukin-6, and other immune mediators, which are increased in portal vein content due to intestinal leakage.[12,13] Zonulin is also an indicator of brain cancer development due to its involvement in the dysfunction of tight junctions in the endothelial cells of the blood–brain barrier.[14]

In the context of CRC, the role of zonulin has been attracting increasing attention. Previous studies have shown elevated serum zonulin levels in patients with CRC.[15] Kushlinskii et al[9] found higher serum zonulin levels in patients with CRC and benign colon tumors compared to healthy controls or patients with inflammatory or irritable bowel disease. However, no relationship was reported between these levels and specific criteria in the TNM cancer staging system, tumor localization, histological structure, and malignancy grade in patients with CRC.

To our knowledge, no previous published literature have compared zonulin levels between CRC and healthy tissues. We examined tissue zonulin levels in our study. Notably, zonulin levels were significantly higher in cancerous tissues; however, in the normal-appearing noncancerous colon segments of patients with CRC, these levels were only slightly higher than those in the control group, which may indicate a potential diagnostic value in the future. Our findings indicate markedly elevated levels of zonulin in cancer tissues, moderately high levels in normal colon tissues among patients with cancer, and comparatively lower levels in colon tissues of the healthy control group, which are significant discoveries.

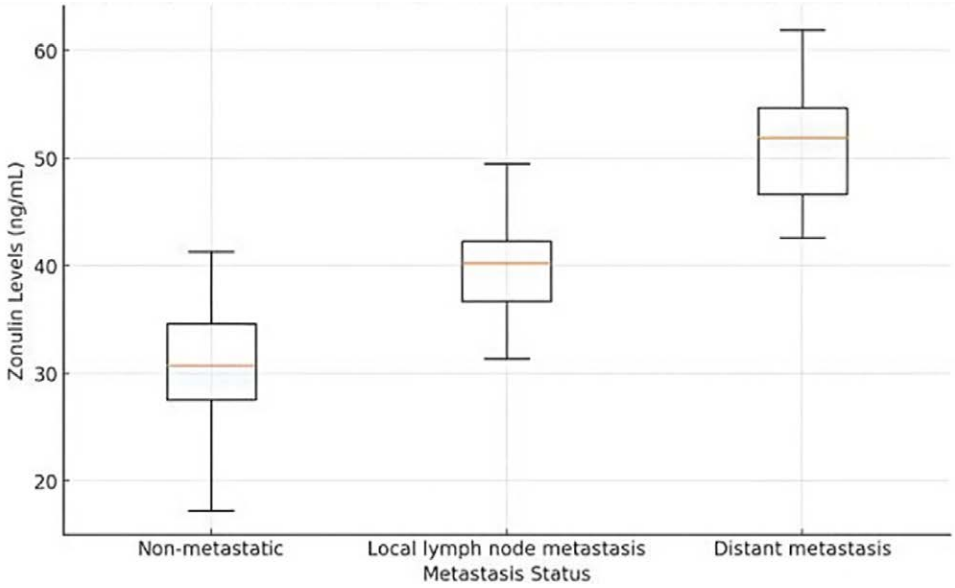

Figure 3. Comparison of zonulin levels in metastatic and nonmetastatic CRC. Box plots showing median and interquartile ranges of zonulin concentrations (ng/mL) in tissues from patients with nonmetastatic disease, local lymph node metastasis, and distant metastasis, demonstrating progressive increase in zonulin levels with advancing metastatic status. CRC = colorectal cancer.

Table 4

Detailed comparison of zonulin levels between right-sided and left-sided colon cancer.

Characteristics	Right-sided CRC (n = 24)	Left-sided CRC (n = 84)	*P*-value*
Healthy tissues (ng/mL)			.003
Mean ± SD	32.20 ± 5.19	29.15 ± 13.36	
Median (IQR)	32.61 (27.70–36.61)	26.70 (21.69–31.44)	
Tumor tissues (ng/mL)			.196
Mean ± SD	56.92 ± 9.96	70.98 ± 30.52	
Median (IQR)	60.98 (49.61–64.27)	63.98 (47.33–91.83)	
Ratio of tumor to healthy tissue zonulin levels			
Mean ratio	1.77	2.43	.015†
Median ratio	1.87	2.40	

CRC = colorectal cancer, IQR = interquartile range, SD = standard deviation.

*Mann-Whitney *U* test.

†Calculated using the ratio of means, *P*-value estimated based on the difference in ratios.

These findings suggest that zonulin may be increased not only in tumor tissue but also in apparently normal adjacent tissues. This suggests that zonulin may reflect changes in the tumor microenvironment and may be a potential biomarker for early diagnosis.[16] Furthermore, we found that zonulin levels differed significantly according to age and sex, a distinction not previously noted in the literature and may need consideration if zonulin levels are to become a prognostic parameter in the future. The difference in zonulin levels between women and men might be explained by the hormonal effects or the differences in the composition of gut microbiota. That is why this finding may present a new view on gender-specific risk factors and pathogenesis of CRC.[17]

Thus, in our study the level of zonulin was significantly higher in the right-sided colon cancers than that in the left-sided scores. The absolute value of zonulin in right colon cancer may be higher than that in left colon cancer due to differences in molecular and pathological characteristics of tumors. This finding could open a new understanding in the terms of the cause for the varied biological characteristic of right and left colon cancer.

Consistent with the findings of Kushlinskii et al,[9] zonulin levels were positively correlated with the treatment modalities administered according to disease stage and metastatic status. The high levels of zonulin observed in metastatic patients indicate the potential role of zonulin in the process of tumor invasion and metastasis. This suggests that zonulin can be used not only as a diagnostic but also as a prognostic biomarker.[18] However, we found no correlation with mortality status, tumor differentiation, or disease localization.

Changes in zonulin levels according to TNM staging suggest that the integrity of the intestinal barrier progressively deteriorates with disease progression. This finding suggests that zonulin may be a potential tool for monitoring disease progression.[19]

The involvement of zonulin in gut innate immunity and its upregulation in several autoimmune diseases, including celiac disease and type 1 diabetes, has been demonstrated.[20–24] In addition, we showed that zonulin expression was upregulated in the intestinal tissues of patients with CRC.

It can be speculated that the dysregulation of zonulin levels and its release may contribute to abnormal barrier regulation, leading to the submucosal passage of environmental non-self-antigens. In this context, increased intestinal permeability might be a consequence of and an initiating or contributing factor in CRC. From this hypothesis, it can be concluded that inflammatory bowel disease, as a potential cause of CRC, could be mediated by zonulin. This may lead to the concept of the prevention and possible medicine or cure for CRC.

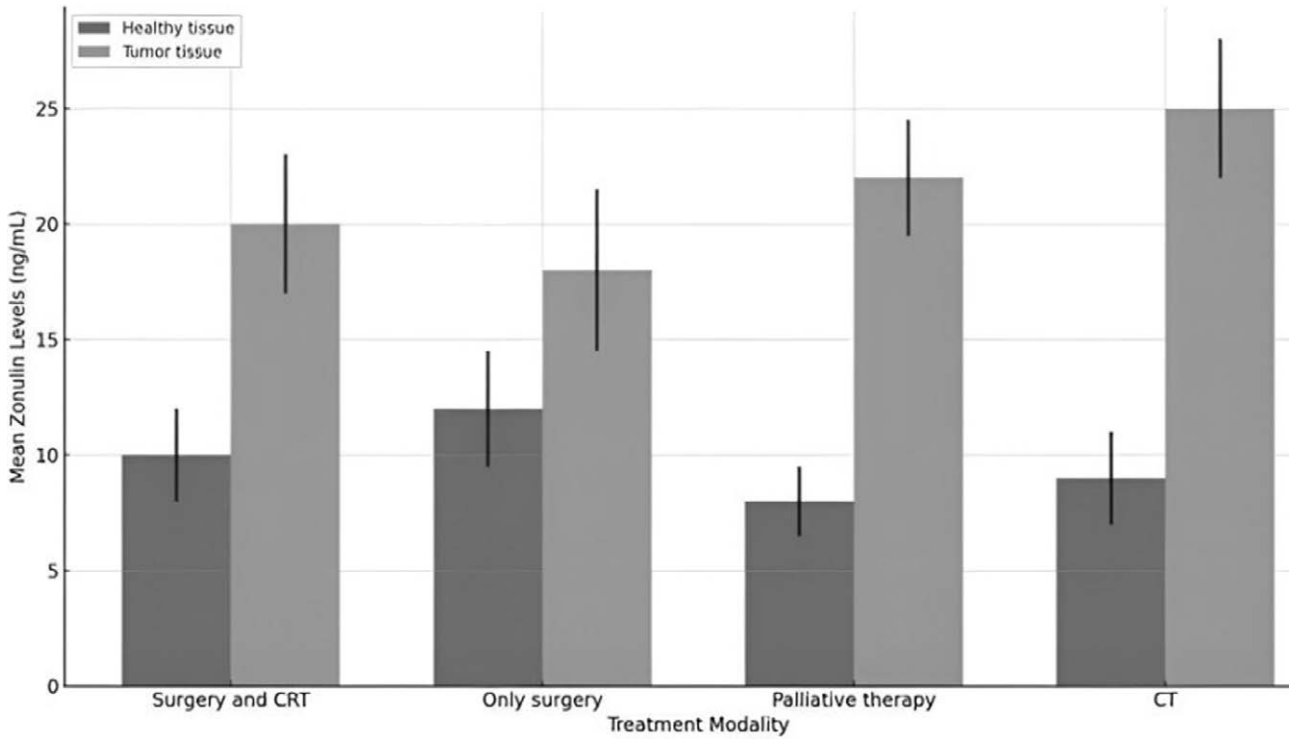

Figure 4. Zonulin levels by treatment modality in healthy and tumor tissues. Bar graph comparing mean zonulin concentrations (ng/mL) with error bars in healthy tissues (dark gray) and tumor tissues (light gray) across different treatment approaches: surgery with chemoradiotherapy (CRT), surgery alone, palliative therapy, and chemotherapy (CT) alone. CRT = chemoradiotherapy, CT = chemotherapy.

In line with the results about the shifts of zonulin levels depending on the treatment modalities, which is presented in the present study, it appears that zonulin can be considered as biomarker of treatment response. In particular, the increase of the zonulin concentration in patients undergoing CT can indicate the influence of CT on the state of the intestinal barrier. This finding may perhaps have implications towards the medical management of the side effects of CT and bettering of CT regimen.

Our study has several limitations that should be acknowledged. First, we did not consider tissue zonulin levels in colon polyps, other mass lesions, or inflammatory diseases such as ulcerative colitis and Crohn's disease. Second, it could be argued that if we had studied tissue and serum zonulin levels together, our contribution to the literature in terms of comparison with other studies on the subject and determining the future roles of zonulin would have been clearer. Third, due to the cross-sectional nature of our study, we were unable to observe how zonulin levels change over the course of the disease. Fourth, our study has an unbalanced gender distribution (78 females vs 30 males), which may affect the reliability of our conclusions regarding gender differences in zonulin levels. Fifth, this is a single-center study, which may limit the generalizability of our findings to different populations. Finally, we did not collect data on the ethnic background of our participants, which could be important given potential variations in gut microbiome composition and intestinal permeability across different ethnic groups. Future longitudinal and multicenter studies with more balanced gender distribution and diverse ethnic representation may help us better understand the role of zonulin in CRC progression.

On the other hand, the strength of our study lies in that all analyses were performed using the same batch number and within 6 weeks after storage of samples at –80°C. This is crucial as zonulin degradation is expected within 1 to 2 months at –20°C, as stated in the manufacturer's instructions. Most other studies in the field have not reported the sample storage conditions, which may explain some of the differences from the present study.

In conclusion, it is found that this study can effectively prove that zonulin may have the prospect as the biomarker of CRC diagnosis, prognosis and treatment. These observations may contribute to the further elucidation of the involvement of zonulin in CRC pathogenesis and objectives of diagnostics and therapies. Therefore, further works should incorporate larger patient numbers and more clinical stages of cancer to provide further insight into the subject of zonulin in cancer.

Author contributions

Conceptualization: Enver Akbas.
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Formal analysis: Enver Akbas.
Funding acquisition: Enver Akbas.
Investigation: Enver Akbas.
Methodology: Gözde Ülfer.
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